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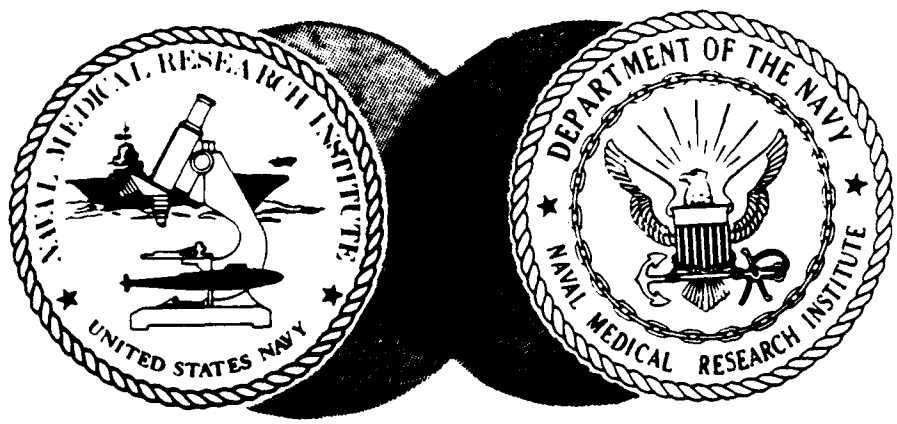

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A TISSUE CULTURE METHOD FOR THE DETECTION OF BACTERIAL ENTEROTOXINS

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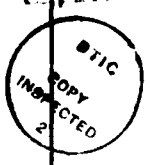
Specific standard steps are described for the implementation of the Y1 mouse adrenal tumor cell line in evaluating the production of bacterial enterotoxins and cytotoxins. The importance of standardization of methods for Y1 cell culture, preparation of test materials, and interpretation of results is emphasized. Rationale for utilization of the Y1 cell assay and difficulties of application are discussed. Adaptations for research purposes and potential applications are described.



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A TISSUE CULTURE METHOD FOR THE DETECTION OF BACTERIAL ENTEROTOXINS

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SUMMARY: Specific standard steps are described for the implementation of the Y1 mouse adrenal tumor cell line in evaluating the production of bacterial enterotoxins and cytotoxins. The importance of standardization of methods for Y1 cell culture, preparation of test materials, and interpretation of results is emphasized. Rationale for utilization of the Y1 cell assay and difficulties of application are discussed. Adaptations for research purposes and potential applications are described.

Key words: Y1 cells; bacterial enterotoxins; *Vibrio cholerae;* enterotoxigenic bacteria; Y1 assay.

I. INTRODUCTION

Since its development by Donta (1), and subsequent adaptation to miniculture by Sack and Sack (2), the Y1 mouse adrenal tumor cell assay has been widely applied in testing clinical, laboratory, and environmental strains for toxigenesis. Considering the acceptance of this system and the different methodologies employed in various laboratories, we believe standard procedures should be adopted. This report describes a specific method for routine titrations of toxins from bacteria of suspected enterotoxigenic potential and recommends additional tests to characterize these toxins.

II. MATERIALS

A. Chemicals and culture media

Medium RPMI 1640, No. 11-306B, Microbiological Associates[1]

$NaHCO_3$, powder, certified ACS, No. S-233, Fisher Scientific[2]

Fetal bovine serum, No. 200-6140, GIBCO[3]

Tryptic soy broth, No. 0370-02-0, Difco[4]

Thioglycollate broth, No. 11720, BBL[5]

Sabouraud dextrose broth, No. 10986[5]

Nutrient broth, No. 11479[5]

Penicillin (5,000 U/ml) streptomycin (5,000 μg/ml), No. 16-700-49, Flow Laboratories[6]

NaOH, 0.2 *N* solution, No. S-274[2]

HCl, 0.2 *N* solution, No. A-52[2]

Dimethyl sulfoxide (DMSO), No. D-128[2]

Trypsin, 1:300, No. 840-7073[3]

Ethylenediaminetetraacetic acid (EDTA), No. E-478[2]

Hanks' balanced salt solution, without calcium, without magnesium, No. 18-104-49[6]

Hanks' balanced salt solution, No. 18-100-49[6]

Cholera enterotoxin, 5 mg protein/ml, No. 8560, Schwarz/Mann[7]

Gentamicin, 50 mg/ml, No. 17-740A[1]

0271-8057/80/0085-0090 $1.50/0

B. Glass and plasticware

Nalgene filter unit, 0.22 μm, No. 245-0045, Nalge Sybron[8]
Media bottles, 500 ml, No. 900-7030[3]
Tissue culture flask, 75 cm², No. 25110-75, Corning[9]
Test tube, 16 × 125 mm, screw-capped, No. 2009-16125, Bellco[10]
96-Well disposable flexible polyvinyl chloride microtitration plates, No. 1220-24B, Dynatech[11]
Disposable pipette tips, No. 77-890-07[6]
96-Well tissue culture cluster, 6.4 mm d well, with cover, sterile, No. 3596, Costar[12]
Pasteur pipettes, disposable, No. 52947-802, VWR[13]

C. Equipment

Test tube rack, No. 2027-16048[10]
Rotary shaker table, No. G2, New Brunswick[14]
Multichannel pipette, 0.050 ml, No. 77-838-00[6]
Microscope, phase-contrast, inverted, Olympus, No, CK-600[13]

D. Cells

Y1 mouse adrenal tumor cells, Dr. Sam Donta[15]

III. PROCEDURE

A. Preparation of complete RPMI 1640 growth medium

1. RPMI 1640: Reconstitute as per manufacturer's instructions adding 2.5 g/l $NaHCO_3$. Filter sterilize through 0.22 μm membrane filter unit and aseptically dispense into sterile 500 ml medium bottles.
2. Fetal bovine serum (FBS): Add to RPMI 1640 to a final concentration of 10%.
3. Sterility tests: Inoculate 1 ml RPMI 1640/10 ml into duplicate tubes of sterile tryptic soy broth (TSB), thioglycollate broth, Sabouraud dextrose broth, and nutrient broth (prepared according to manufacturer's instructions). Incubate for 7 d at 37° C and examine for evidence of contamination.
4. Antibiotics: Add 100 IU/ml penicillin and 100 μg/ml streptomycin from sterile stock solution to RPMI 1640 before use.
5. pH: Adjust pH of RPMI 1640 to 7.4 with sterile 0.2 *N* NaOH or 0.2 *N* HCl.

B. Cell culture storage

1. Cryopreservation: Freeze 1 ml aliquots of the Y1 adrenal cells in RPMI 1640 complete growth medium with 7.5% vol/vol dimethyl sulfoxide at 5×10^6 cells/ml following established methods (3). Store in liquid nitrogen.
2. Recovery: Thaw rapidly by vigorously agitating vial in a 37° C water bath. Establish cells in 20 volumes complete RPMI 1640 in a 75 cm² tissue culture flask and incubate at 37° C in a humidified, 95% air: 5% CO_2 atmosphere. Replace medium after attachment of cells to substrate (approx 4 h).

C. Cell subculture

When the tissue culture layer is approximately 80% confluent, renew complete growth medium and subculture after 12 to 24 h. Dissociate with 0.05% trypsin and 1:3000 wt/vol EDTA in Ca^{++}, Mg^{++}-free Hanks' balanced salt solution. Passage cells at a ratio of 1:2 to 1:32 (depending on day of usage) for stock cultures or count cells by trypan blue dye exclusion, using a hemocytometer (4), and dilute to appropriate cell concentration (5) for microtiter toxin assays (*see* Section E). Incubate as in Section B.

D. Preparation of test supernatant solutions

1. Inoculate each bacterial strain to be tested, e.g., *Vibrio cholerae,* into 8 ml of TSB or Syncase broth (6) in 16 × 125 ml screw-capped glass test tubes. Eight specimens can be assayed per 96-well microtiter plate.
2. Incubate cultures and an uninoculated broth control for 18 to 24 h at 30° C, slanting tubes in a test tube rack on a rotary shaker at 150 rpm.
3. Centrifuge cultures at 2600 ×*g* for 25 min.
4. Draw off supernatant solutions for assay (or store at 4° C).
5. Dispense 50 μl of Hanks' balanced salt solution (HBSS) into all wells in Columns 2 to 11 of a disposable polyvinyl chloride (PVC) microtitration plate.
6. Dispense 100 μl of each bacterial supernatant solution to be tested into a well in Column 1 and label the row to identify each strain.
7. Using the 50 μl multichannel pipette with eight sterile, disposable tips, make serial twofold dilutions of the test supernatant solutions by transferring 50 μl from each well in Column 1 to Column 2. Mix by withdrawing and aspirating the dilution twice. Similarly continue diluting to Column 11 by transferring 50 μl of each sample to the next Column and mixing, discarding the excess 50 μl from Column 11.
8. In Column 12, add 50 μl of RPMI 1640 (tissue culture media control) to Wells A and

B, add 50 μl of HBSS (diluent control) to Wells C and D, add 50 μl of uninoculated broth (bacteria culture medium control) to Wells E and F, and add 50 μl of cholera enterotoxin (CT) diluted to 4 ng/ml (200 pg/well) in HBSS (toxin positive control) to Wells G and H.

E. Toxin assay

1. Prepare 96-well, tissue culture cluster, microtiter plate cell cultures by dispensing into each well 0.2 ml of complete RPMI 1640 growth medium containing the following concentrations of Y1 cells to produce suitable cultures for performing assays on subsequent days: 1×10^5 cells/well for Day 1, 5×10^4 cells/well for Day 2, 1×10^4 cells/well for Days 3 or 4.
2. Incubate as for stock cultures (Section B) until cells reach 50 to 80% confluency.
3. Aspirate medium from the wells, using a sterile, flame-polished Pasteur pipette connected, by means of a trap flask, to a vacuum pump adjusted to achieve a pressure no greater than 50 mm Hg.
4. Using the multichannel pipette and sterile disposable tips, transfer samples in each column from disposable PVC to tissue culture microtitration plates, starting with Column 12. Replace tips and transfer Columns 11 through 1 with a single set of tips working from the highest dilutions to the undiluted supernatant solutions.
5. Incubate at 37° C for 5 min as described in Section B.
6. Aspirate the samples from the wells.
7. Wash cells by filling all wells with HBSS and aspirating as in Step 3 above. Repeat cell wash.
8. Dispense 0.2 ml RPMI 1640 complete growth medium containing 100 μg/ml gentamicin into each well.
9. Incubate plates for 24 h (*see* Step 5 above).

F. Recording of results

1. Scan cells at 40X under a phase-contrast inverted microscope to determine the percentage of cells affected and at 100X for typical refractile rounded morphology [cytotonic (1,7)] or for a granular, crenated, or lysed appearance [cytotoxic (7-9)]. *See* Fig. 1.
2. Score individual wells as 1+ to 4+, corresponding to 25%, 25 to 50%, 50 to 75%, or 75 to 100% of cells exhibiting cytotonic morphology. Similarly, use 1+ to 4+ to designate corresponding cytotoxic morphology.

IV. DISCUSSION

In order to minimize nonspecific rounding of the cells, sometimes noted in negative control wells, proper cell maintenance is required. Additional Y1 cell culture descriptions and recommendations for handling have been published elsewhere (10). Although Ham's medium F10 with 15% horse serum and 2.5% FBS as specified in the original assay procedures (1) and other media (10) are adequate for Y1 cell growth, we have found medium RPMI 1640 with 10% inactivated FBS yields consistent results. Medium F10 possesses only a minimal buffering capacity, requiring more frequent medium renewal when maintaining cultures. Some lots of commercial horse serum exhibit cell toxicity and usually contain higher levels of cyclic adenosine-3′:5′ monophosphate (cAMP) than FBS (11). The involvement of cAMP in the mechanism of action of bacterial toxins has been reviewed elsewhere (12). We routinely screen lots of FBS for ability to promote clonal growth of secondary hamster embryo cell cultures before inclusion in RPMI 1640 Y1 medium. Procedures for maintaining serum quality control have been published (13). We have found frozen storage of Y1 cells to be a more convenient and consistent method for making available cells with an acceptable level of nonspecific rounding (<5%) than regular passage, especially if cultures are needed at irregular intervals. Cryopreservation, using glycerol as a substitute for DMSO or other freezing methods, can be substituted for those described. Cultures are always refed 1 d before subculture, split before confluency, and passed at least once before use.

The use of 96-well, microtiter plates may cause some difficulties. Removing the polystyrene plates from their polyvinyl sleeves generates a static charge on the plates, especially under low humidity conditions. The static charge can prohibit even distribution of cells on the growth surfaces. Storage of plates in a humidified incubator prior to seeding and passage of the plate through a degaussing coil after seeding can alleviate this problem. However, some migration of cells to the periphery of the outer wells prior to cell attachment, can, depending on seeding density, limit those cells suitable for toxin assays to the centermost 60 wells. Other plastic tissue culture vessels, such

as 24-well clusters or 60 mm dishes, can be used to obtain equivalent results by scaling up the number of cells and fluid volumes employed in proportion to growth surface areas.

Other aspects of the assay itself are important to mention if reliable results are to be obtained. When aspirating medium, test supernatant solutions, or HBSS from the culture surface, use a minimum vacuum and always aspirate from the same side of the well to minimize disruption of the cell layer. Keep exposure time of the cells to the air to <1 min, that is, limit each operation to three columns at a time. It is convenient to set up test supernatant solutions, serial dilutions, controls, et cetera, in inexpensive, disposable, 96-well polyvinyl plates well ahead of time, and make transfers, using a multichannel pipetter and disposable tips, to Y1 cells. Avoid cross-contamination by using fresh tips for transfer and washing and by rinsing the aspirating pipette with 70% ethanol between wells (or by proceeding from low to high concentrations with serial dilutions).

Controls must be run for every plate, including uninoculated broth, HBSS, and RPMI 1640 as negative controls and an appropriate parallel toxigenic culture supernatant solution, viz. *Vibrio cholerae* 569B (ATCC 25870) or *Escherichia coli* H10407 (14), and purified CT for positive controls. If assays on the supernantant solutions cannot be made immediately, storage at 4° C for up to a month should not result in loss of activity. Inclusion of gentamicin in the final culture medium is sufficient for inhibition of bacterial growth. We have not found gentamicin resistance to be common among *V. cholerae, E. coli,* or *Aeromonas hydrophila* cultures we have examined in our laboratory. In laboratories where tissue culture media are prepared infrequently or where space necessitates culturing bacteria or mycoplasma in proximity to cell culture facilities, the stability and antibiotic spectrum of gentamicin may indicate its inclusion in all tissue culture media.

Although it is difficult to define bacterial culture conditions that will yield optimal toxin

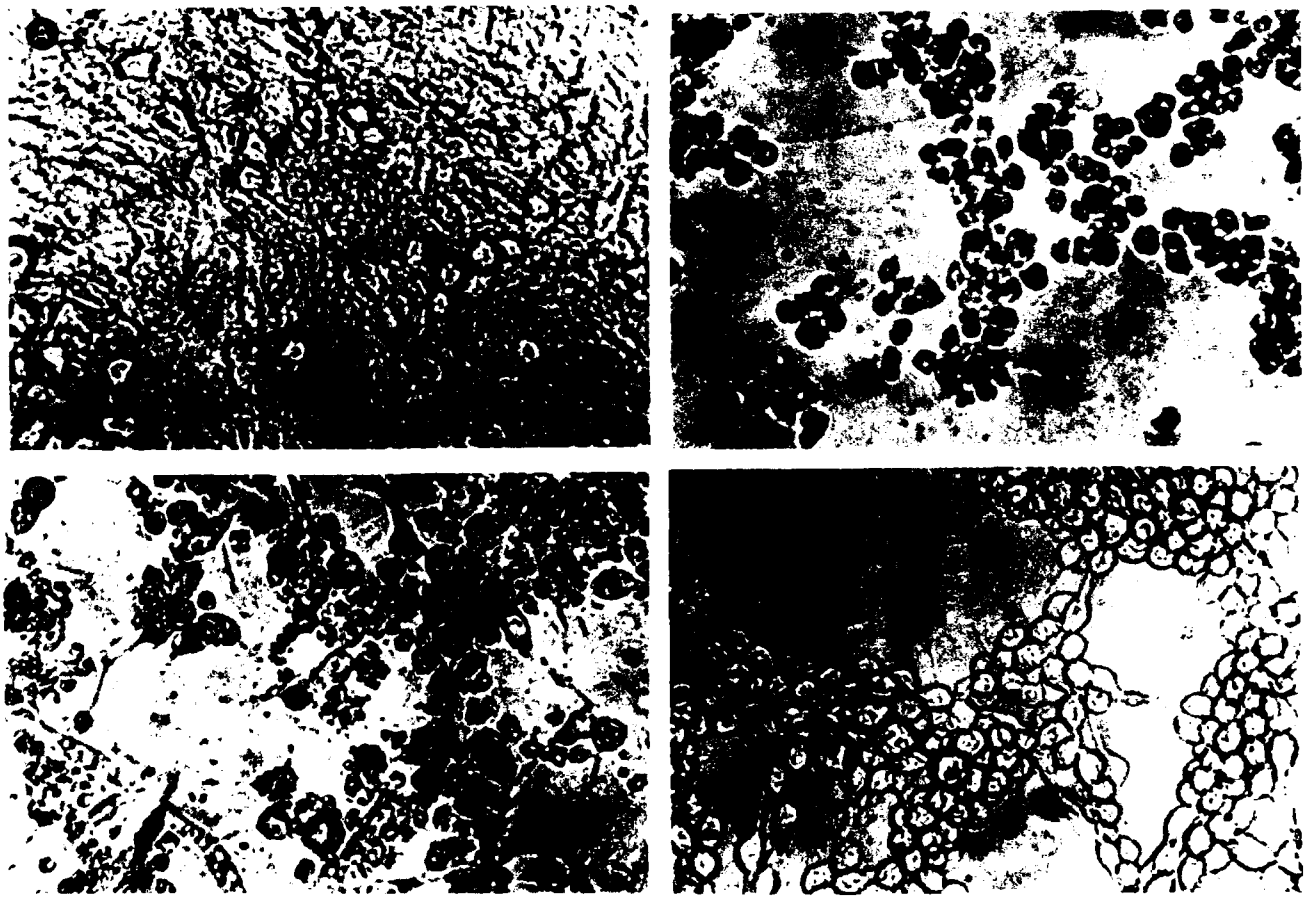

FIG. 1. Morphological appearance at 24 h of cells exposed to bacterial supernatant solutions. *A*, Negative control; *B*, cytotoxic response; *C*, combined cytotoxic and cytotonic response; *D*, cytotonic response. Inverted light microscopy. ×74.

production by all strains, the conditions outlined above are satisfactory for routine screening of isolates. Media containing additional carbohydrate, or of reduced buffering capacity, will result in production of excess acid, i.e., a pH as low as 4, which is detrimental to cell cultures and toxin stability. Media of osmolarities markedly different from RPMI 1640 can also distort results. Enhancement of toxin production with certain strains by modification of culture conditions (media, temperature, time, et cetera) provides some specific improvements, but these are not necessarily applicable to all cultures. Such parameters should be considered by investigators interested in specific organisms, along with other methods such as lincomycin incorporation into the medium, polymyxin release, animal passage, or membrane disruption, for enhancement of toxin production.

Interpretation of Y1 assay results should be based on considerations as follows. Many researchers consider only rounding of >50% of the cells as a positive result for enterotoxin (3+ – 4+). Hence, we have chosen a CT concentration (200 pg/well) for the positive controls to reflect such criteria. Our use of available Y1 cells and the outlined cell maintenance procedures have kept baseline rounding to <5% and allowed reproducible detection of enterotoxin at levels as low as 5 pg/well by comparing negative (Fig. 1*A*) and positive test cell rounding.

Many bacterial cultures induce cytotoxic changes in Y1 cells that are difficult to differentiate morphologically from those caused by CT. Cytotoxin-exposed cells (Fig. 1*B*) usually will not exclude trypan blue dye (9), and often lift off the substrate, whereas CT-treated cells (cytotonic) remain viable after washing and medium renewal. In some strains where both activities are expressed (Fig. 1*C*), cytotoxic masking of a cytotonic response (Fig. 1*D*) may be reduced at higher dilutions. Some investigators have reported inhibition of cytotoxic components by limited heat treatment of the supernatant solution (8,9). Addition of protease inhibitors (9,15,16) to the test samples, inclusion of ammonium sulfate in the bacterial media (17), or gel filtration fractionation of supernatant solutions or ultrafiltration concentrates may inhibit or remove cytotoxins. Whereas the nature of most cytotoxins is unclear, some have been linked to pathological changes observed in animal model test systems (9,18) and should not be disregarded. HeLa or similar cell lines, which lack the specific cAMP elevation and steroidogenic responses of Y1 cells, can be used to differentiate between CT-like and other bacterial toxins (7,9). Detection of steroids in Y1 cell culture media indicates a CT-like mode of action (1). Inhibition of Y1 response by sample preincubation with purified cholera antitoxin (19) or the cell enterotoxin receptor, G_{M1} ganglioside (20), indicates an antigenic or binding similarity, respectively, between the test sample and CT. Other immunological methods, such as enzyme-linked immunosorbent assay (ELISA) (21,22) or radioimmunoassay (RIA) (23), are available for sensitive detection of antigenic relatedness to CT components.

The methods outlined here have been used to screen several hundred environmental strains of *V. cholerae*, *E. coli*, and *Aeromonas* isolated in our laboratory at the University of Maryland and to characterize a large number of clinical isolates of *V. cholerae* obtained from other laboratories. The method described herein has been used for over 4 yr and has provided high sensitivity, interlaboratory reproducibility, and good correlation with other toxin assays. It is recommended for use in laboratories with tissue culture capability where it is necessary to screen large numbers of organisms for toxigenic characterization.

CAUTION: Cholera enterotoxin is a hazardous material. Control and test bacterial strains must be handled as potential pathogens, and applicable laboratory safety precautions must be taken.

V. REFERENCES

1. Donta, S. T.; King, M.; Sloper, K. Induction of steroidogenesis in tissue culture by cholera enterotoxin. Nature (New Biol.) 243: 246–247; 1973.
2. Sack, D.; Sack, R. B. Test for enterotoxigenic *Escherichia coli* using Y1 adrenal cells in miniculture. Infect. Immun. 11: 334–336; 1975.
3. Schroy, C. B.; Todd, P. A simple method for freezing and thawing cultured cells. Procedure 76035. TCA Manual 2: 309–310; 1976.
4. Tolnai, S. A method for cell viability count. Procedure 70151. TCA Manual 1: 39–40; 1975.
5. Freeman, A. Tissue culture mathematics. Procedure 99115. TCA Manual 1: 239–241; 1975.
6. Finkelstein, R. A.; Atthasampunna, P.; Chulasamaya, P.; Charunmethee, P. Pathogenesis of experimental cholera: biological activities of purified procholeragen A. J. Immunol. 96: 440–449; 1966.
7. Keusch, G. T.; Donta, S. T. Classification of enterotoxins on the basis of activity in cell culture. J. Infect. Dis. 131: 58–63; 1975.
8. Donta, S. T.; Haddow, A. D. Cytotoxic activity of *Aeromonas hydrophila*. Infect. Immun. 21: 989–993; 1978.
9. Cumberbatch, N.; Gurwith, M. J.; Langston, C.; Sack, R. B.; Brunton, J. L. Cytotoxic enterotoxin produced by *Aeromonas hydrophila:* relationship of toxigenic isolates to diarrheal disease. Infect. Immun. 23: 829–837; 1978.
10. Schimmer, B. P. Adrenocortical Y1 cells. Jakoby, W. B.; Pastan, I. H. eds. Methods in enzymology, vol. 58. New York: Academic Press; 1979: 570–574.
11. Fox, C. H.; Sanford, K. K. Chemical analyses of mammalian sera commonly used as supplements for tissue culture media. Procedure 94121. TCA Manual 1: 233–237; 1975.

12. Vaughan, M.; Moss, J. Mechanism of action of choleragen. J. Supramol. Struct. 8: 473-488; 1978.
13. McGarrity, G. J. Serum quality control. Procedure 75129. TCA Manual 1: 167-169; 1975.
14. Donta, S. T.; Moon, H. W.; Whipp, S. C. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. Science 183: 334-336; 1974.
15. Callahan, L. T., III. *Pseudomonas aeruginosa* enterotoxin: purification by preparative polyacrylamide gel electrophoresis and the development of a highly specific antitoxin serum. Infect. Immun. 14: 55-61; 1976.
16. Schnebli, H. P.; Burger, M. M. Selective inhibition of growth of transformed cells by protease inhibitors. Proc. Natl. Acad. Sci. USA 69: 3825-3827; 1972.
17. Liu, P. V.; Hsieh, H. Inhibition of protease production of various bacteria by ammonium salts: its effect on toxin production and virulence. J. Bacteriol. 99: 406-413; 1969.
18. Seidler, R. J.; Allen, D. A.; Lockman, H.; Colwell, R. R.; Joseph, S. W.; Daily, O. P. Isolation, enumeration and characterization of *Aeromonas* from polluted waters encountered in diving operations. Appl. Environ. Microbiol. 39: 1010-1018; 1980.
19. Evans, D. J., Jr.; Ruiz-Palacios, G.; Evans, D. G., DuPont, H. L.; Pickering, L. K.; Olarte, J. Humoral immune response to the heat-labile enterotoxin of *Escherichia coli* in naturally acquired diarrhea and antitoxin determination by passive immune hemolysis. Infect. Immun. 16: 781-788; 1976.
20. Donta, S. T.; Viner, J. P. Inhibition of the steroidogenic effects of cholera and heat-labile *Escherichia coli* enterotoxins by G_{M1} ganglioside: evidence for a similar receptor site for the two toxins. Infect. Immun. 11: 982-985; 1974.
21. Holmgren, J.; Svennerholm, A. M. Enzyme-linked immunosorbent assays for cholera serology. Infect. Immun. 7: 759-763; 1973.
22. Yolken, R. H.; Greenberg, H. B.; Merson, M. H.; Sack, R. B.; Kapikian, A. Z. Enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. J. Clin. Microbiol. 6: 439-444; 1977.
23. Hejtmancik, K. E.; Peterson, J. W.; Markel, D. E.; Kurosky, A. Radioimmunoassay for the antigenic determinants of cholera toxin and its components. Infect. Immun. 17: 621-628; 1977.

[1] Microbiological Associates, Bethesda, MD.
[2] Fisher Scientific Co., Fair Lawn, NJ.
[3] Grand Island Biological Co., Grand Island, NY.
[4] Difco Laboratories, Detroit, MI.
[5] Baltimore Biological Laboratories, Cockeysville, MD.
[6] Flow Laboratories, Rockville, MD.
[7] Schwarz/Mann, Orangeburg, NJ.
[8] Nalge Co. Div. Sybron, Rochester, NY.
[9] Corning Glass Works, Corning, NY.
[10] Bellco Biological, Vineland, NJ.
[11] Dynatech Laboratories, Alexandria, VA.
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Approved by Author

David R Maneval, Jr. May 29, 1981
Signature Date